

AMENDMENT

To the Drawings

The drawings are amended as indicated by marks in red ink on original Figures 2 (now 2A and 2B), 3 (now 3A, 3B, and 3C), 4A (now 4A and 4B), 5, 6, 13 (now 13A and 13B), and 16.

To the Specification

Please amend the following paragraphs of the specification to read as follows. (A marked up version of the amended paragraphs is attached at the end of this Amendment and Response.)

Paragraph on page 6, lines 6-8

Figures 2A and 2B each depict the results of DNA/DNA and DNA/PNA hybridization experiments at various temperatures and concentrations developed by chemiluminescence.

Paragraph on page 6, lines 9-11

Figures 3A, 3B and 3C show the results of a hybridization experiment between two restriction digests of pBR322 with a labeled PNA probe separated on three different electrophoretic gel systems: non denaturing 4-20% acrylamide (Figure 3A); 7 M urea 6% acrylamide (Figure 3B); and 1% agarose (Figure 3C).

Paragraph on page 6, lines 12-13

H3
Figures 4A and 4B show the effect of temperature in a hybridization experiment between double stranded DNA pBR322 and labeled PNA probe.

Paragraph on page 6, lines 18-20

H4
Figure 5 is a plot comparing the stability of complementary (trace identified by ♦), one-base mismatch (trace identified by ■) and two-base mismatch (trace identified by ●) PNA/DNA duplexes in a denaturing medium at various temperatures.

Paragraph on page 6, lines 21-22

Figure 6 is a plot comparing the stability of complementary (trace identified by ♦) and two-base mismatch (trace identified by ■) PNA/DNA duplexes at various temperatures.

Paragraph on page 7, lines 12-13

Figure 13A is a schematic representation of the sample loading process with an offset pinched injector.

Figure 13B is a schematic representation of the electrophoresis separation process with an offset pinched injector.

Paragraph on page 18, line 20 to page 19, line 8

With reference to figures 2A and 2B, a P indicates that a PNA oligomer probe was annealed (hybridized) and a D indicates that a DNA oligomer probe was annealed (hybridized) to the nucleic acid duplex. M indicates a marker lane. Lanes A1, B1, C1, D1 and E1 exhibit bands within the gel which represent the PNA probe/nucleic acid duplex which has migrated into the gel and is detected. Because each sample was exposed to differing conditions of temperature during the hybridization, the experiment demonstrates that the annealing phenomenon is fairly temperature independent. Under identical conditions, the analogous DNA probe/nucleic acid duplex is barely detectable; as indicated by lanes A2, B2, C2, D2, and E2. In each of these lanes, the labeled DNA probe is observed as a large circular blob at the bottom of each lane. This blob is visible because the excess DNA oligomer is charged and therefore migrates in the gel. The blob is at the bottom of the gel because it is small (15 nucleotides) and moves through the gel very rapidly. Conversely, the lanes with PNA probe exhibit background only near the loading well since the probes migrate into the gel primarily by passive diffusion. Detection of the PNA/nucleic acid duplex in lanes 3 and 5 of all samples required a longer exposure time for the film in order that the band be observed. Thus, there is a concentration dependence whereby the limits of detection are dependent on the starting amount of target DNA and the amount of probe

available to bind to the target sequence. The controls demonstrate that the sample DNA/DNA melts even at 55°C which is also a suitable annealing temperature for DNA/PNA duplexes. Moreover, no annealing temperature need be attained since duplex is formed for control F1.

Paragraph on page 19, line 29 to page 20, line 12

With reference to figures 3A, 3B and 3C, samples were only loaded on the gels where indicated by a numeral 1, 2, or 3. All numeral 1's were 4 ng of pBR322 digested with BstNI. All numeral 2's represent the higher concentration of 16 ng of pBR322 digested with Mspl. Similarly, all numeral 3's represent the less concentrated 4 ng of pBR322 digested with Mspl. The letter M indicates that a size marker was run in those lanes. As would be expected, for the different gel conditions, the mobility of the components varies substantially from gel to gel. With reference to figure 3A, lane 1 contains a single stranded DNA fragment that is 939 base pairs (bp) long. The same band is apparent in lane 1 of figure 3C. It is however, not seen in figure 3B. This is because the fragment is too large to be successfully electrophoresed through 6% acrylamide. With regard to the PNA/fragment complex found in lane 2 of figures 3A, 3B, and 3C there is one fragment of approximately 97 bp band that is visible. Again it is clear that different gels impart different mobilities on fragments of the same length. Lane 3 found in figures 3A, 3B, and 3C, which is a more dilute solution of the fragment/PNA complex seen in lane 2, runs in the same part of each respective gel system.

Paragraph on page 20, line 23 to page 21, line 12

Hb
Six sets of reactions were set up in duplicate. 4 ng of linearized double stranded pBR322 was used for each reaction. To the pBR322 was added 2pmol of a biotinylated PNA, 1/10th vol of TE buffer and dH₂O to bring the reaction volume to 10 µL. Sets of samples were incubated at the indicated temperature for 10 minutes, and then slowly (over 30 minutes) cooled to room temperature. (Sample 2B was heated initially at 94°C, sample 3B and D heated at 85°C, sample 4B and D -75°C, sample 5B and D -65°C, sample 6B and D -55°C, and sample 7B and D were left at room temperature.) After all samples had slowly cooled to room temperature, dH₂O,

1/10th volume of medium salt (NEB 2- 10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiotheritol) and 1 ml of Pst I restriction enzyme were added to each sample. These were incubated at 37°C for 20 minutes and then left overnight at ambient temperature. The next day, each tube was given an additional 0.5 µL of restriction enzyme and incubated at 37°C for 15 minutes. With reference to figure 4, one set of samples (B) had loading dye added and were ready to load on the agarose gel. The other set (D) was incubated at 94°C for 10 minutes. Then loading dye was added to the D set and the samples were loaded. All samples were electrophoresed through a 1% agarose gel. The components in each gel were then transferred by capillary action onto a non-charged nylon membrane. The next day the membrane was dried and crosslinked with UV (ultraviolet light, in which the source delivers a total energy exposure of 33,000 µjoules/cm².) The membranes were placed in a heat-sealable bag and the biotin label was detected using a chemiluminescent kit (# 7006) purchased from New England Biolabs. The manufacturer's directions were followed for this detection. The membrane was exposed to X-ray film and developed in order to attain the image.

Paragraph on page 22, line 23 to page 23, line 6

KG

Referring to figure 4A, a very efficient discrimination was observed even though stringent conditions could not be applied through controlled temperature. The intensities of the bands from the samples provided by the carrier, lanes 3 and 4, were approximately half the intensity of the band resulting from the wild type PNA/DNA hybrid, lane 1. The mutant PNA probes did not hybridize with the wild type DNA sample, lane 2, demonstrating the high specificity of the method as applied to single base pair discrimination of the sample DNA. In all of the lanes, excess labeled PNA probe was observed with varied intensity, the most intense being observed in lane 2, where the mutant PNA probe did not form detectable amounts of hybrid with the wild type DNA sample. The efficiency in single base pair discrimination observed are due to two factors: 1) the labeled PNA probes are intrinsically highly specific : and 2) the relatively short length of the PNA probes, and most importantly only a small excess of probe is used for the assay. This is in contrast to the traditional Southern blotting in which a high

excess of (DNA) probe is normally used. When an excess of the mutant PNA probe was used with the wild type DNA sample, a false positive was observed, lane 5, figure 4B.

Paragraph on page 28, lines 9-14

In operation and referring to figure 13A, a sample is added to the injection zone 24, and a target, if present, will hybridize with probe 28. A high voltage (HV) is applied to the zones 16, 24 and 30 relative to the ground (GND) applied to zone 26 and is controlled to pinch off the flowing sample stream, preventing diffusion of sample 22 into the separation channel 20 providing an injection volume which is independent of sampling time. The electrical potential between zones 16, 24, 30 and 26 is then shut off, and a potential is imposed between zone 16 and zone 30 to separate the components of the sample by size and electrophoretic mobility, for example, components 34 and 38 shown in figure 13B.

Paragraph on page 31, lines 14-23

With reference to figure 16, the size of the hybrid of the fluorescein labeled PNA and the target DNA fragment can be detected. Since the PNA is labeled with fluorescein, the PNA Probe is carrying a negative charge which comes from the negatively charged fluorescein. As a result, the fluorescein labeled PNA will move in the capillary but not as fast as DNA or PNA/DNA hybrids, towards the anode in an electric field when it is injected from the cathode. Figure 16 shows an electropherogram of a PNA probe hybridization determination of pBR322 DNA Msp1 and BstN1 digests. The hybridized PNA and unhybridized PNA were separated under 704 V/cm over a distance of 22 mm to the detector. The formation of PNA/Msp1 fragment (97 bases) hybrid duplex and PNA/BstN1 fragment (929 bases) hybrid duplex are detected within 30 seconds. The PNA in excess is detected after 40 seconds. The fragments containing the complementary sequence were separated and specifically detected by LIF within 30 seconds.